Dipentyl Phthalate Dosing during Sexual Differentiation Disrupts Fetal Testis Function and Postnatal Development of the Male Sprague-Dawley Rat with Greater Relative Potency than Other Phthalates

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Phthalate esters (PEs) constitute a large class of plasticizer compounds that are widely used for many consumer product applications. Ten or more members of the PE class of compounds are known to induce male fetal endocrine toxicity and postnatal reproductive malformations by disrupting androgen production during the sexual differentiation period of development. An early study conducted in the rat pubertal model suggested that dipentyl phthalate (DPeP) may be a more potent testicular toxicant than some more extensively studied phthalates. Regulatory agencies require dose-response and potency data to facilitate risk assessment; however, very little data are currently available for DPeP. The goal of this study was to establish a more comprehensive data set for DPeP, focusing on dose-response and potency information for fetal and postnatal male reproductive endpoints. We dosed pregnant rats on gestational day (GD) 17 or GD 14–18 and subsequently evaluated fetal testicular testosterone (T) production on GD 17.5 and GD 18, respectively. We also dosed pregnant rats on GD 8–18 and evaluated early postnatal endpoints in male offspring. Comparison of these data to data previously obtained under similar conditions for di (2-ethylhexyl) phthalate indicates that DPeP is approximately eightfold more potent in reducing fetal T production and two- to threefold more potent in inducing development of early postnatal male reproductive malformations. Additionally, fetal testicular T production was more sensitive to inhibitory effects of DPeP exposure than was gene expression of target genes involved in male reproductive development, supporting the use of this endpoint as a critical effect in the risk assessment process.

Key Words: antiandrogen; risk assessment; fetal male rat endocrine; dipentyl phthalate.

Phthalate esters (PEs) are found in many consumer products including personal care products, pharmaceuticals, cosmetics, food packaging, and medical products. Phthalates used as plasticizers are not chemically bound to the plastic polymers and can therefore leach out and become available for human exposure or environmental contamination. Consequently, toxicity of these compounds is a concern for human health, and appropriate assessment of the risk associated with exposure to these compounds is of high importance.

Animal research has demonstrated adverse effects of phthalate exposure during sexual differentiation on the development of the male reproductive system. *In utero* exposure during this critical gestational period to phthalates such as di (2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP) results in malformation of reproductive tissues in males including reduced anogenital distance (AGD), nipple retention, presence of a vaginal pouch, cleft phallus, hypospadias, epididymal agenesis, undescended testes, and reduced accessory sex gland size (Gray *et al.*, 2000; Mylchreest *et al.*, 1998, 1999). Phthalates do not bind the androgen receptor but instead are antiandrogenic through disruption of testosterone (T) synthesis. Decreased androgen production caused by phthalate exposure during the sexual differentiation period is causally linked to phthalate-induced malformations (Barlow and Foster 2003; Mylchreest *et al.*, 1998, 2000; Parks *et al.*, 2000). Phthalate exposure during the gestational day (GD) 14–18

period has also been linked to decreased insulin-like hormone three (insl3) gene expression, which is associated with gubernacular malformations in male rats (Wilson *et al.*, 2004).

Currently, the U.S. Environmental Protection Agency (U.S.EPA) Integrated Risk Information System human health assessment program is focusing on risk assessment for six phthalates including DEHP, DBP, di-isononyl phthalate, benzylbutyl phthalate (BBP), diisobutyl phthalate (DIBP), and dipentyl phthalate (DPeP) (U.S.EPA, 2009). Recently, these phthalates, along with diisodecyl phthalate and di-n-octyl phthalate, were also included on the U.S.EPA Office of Chemical Safety and Pollution Prevention's (EPA OCSPP) Chemical Action Plan (http://www.epa.gov/oppt/existingchemicals/pubs/ecactionpln.html) to "enhance the Agency's current chemicals management program" on the phthalates and other chemicals.

There is a wealth of rodent data available related to the antiandrogenic actions of some phthalates. However, very little data are available regarding the reproductive toxicity of DPeP. In an early study performed in the pubertal male rat model, DPeP induced testicular toxicity with the greatest potency of the phthalates tested in the study (Foster *et al.*, 1980). Additional studies in the pubertal rat demonstrated morphological changes in the testes as soon as 6 h following administration of DPeP (Creasy *et al.*, 1983; Foster *et al.*, 1982) and inhibition of testicular steroidogenic enzymes at 16 h and 2 and 4 days following DPeP dosing (Foster *et al.*, 1983). In the fetal rat model, we previously demonstrated that DPeP *in utero* exposure throughout GD 14–18 was approximately threefold more potent in reducing testicular T production than were DEHP, BBP, DBP, or DIBP exposures during the same period (Howdeshell *et al.*, 2008). Nevertheless, more extensive fetal T production, gene expression, and postnatal dose-response data are required to definitively characterize the potency of DPeP relative to other phthalates deemed positive for antiandrogenic activity. The current study was designed to generate these data, which also can be used to conduct a phthalate cumulative risk assessment.

We previously demonstrated that fetal T production is more sensitive to the inhibitory effects of phthalates than are gene expression of insl3 and the androgen synthesis-related genes, StAR and Cyp11a (Howdeshell *et al.*, 2007b, 2010). Lehmann *et al.* (2004) demonstrated similar relative sensitivities of total extracted testicular T on GD20 and gene and protein expression of StAR and SR-B1 in male offspring of dams dosed daily with DBP during GD 12–19. Nevertheless, total testicular T was not more sensitive than fetal testis T production to disruption by *in utero* DBP exposure from GD 8 to 18 when T was extracted on GD 18 (Howdeshell *et al.*, 2008). The GD of necropsy in these two studies was 2 days apart, which may contribute to the sensitivity differences. Therefore, to clarify conflicting reports related to sensitivity of phthalate-induced disruption of T production, total extracted T, or testicular gene expression, additional dose-response data are required. Based on effects measured in DBP-exposed fetuses, we predicted that DPeP

would reduce fetal testicular T production at lower dosage levels than those that reduced fetal testis gene expression levels of StAR, Cyp11a, and insl3.

The current study addresses the hypotheses that (1) DPeP is more potent than DEHP for inducing male reproductive developmental toxicity during fetal and neonatal life and (2) a reduction in fetal T occurs at lower dosage levels of DPeP than do reductions of insl3, StAR, and Cyp11a gene expression levels. To test these hypotheses, we performed a series of dose-response experiments to assess both fetal and postnatal effects of DPeP and we compared the effective dose to 50% of the tested population (ED50)s with those of DEHP (Gray *et al.*, 2009; Howdeshell *et al.*, 2008). We assessed fetal T production following DPeP exposure throughout the sexual differentiation period, as well as following a single dose of DPeP administered within this period. We determined that DPeP reduces fetal testicular T production and induces early postnatal male reproductive malformations with eightfold and two- to threefold (respectively) greater potency than DEHP. We additionally demonstrated that T production was the most sensitive fetal endpoint tested to *in utero* DPeP exposure and would therefore likely be the most appropriate endpoint to use in the risk assessment of PEs.

MATERIALS AND METHODS

Animals. Timed-pregnant Sprague-Dawley (SD) rats were purchased from Charles River Breeding Laboratory (Raleigh, NC) and Harlan Laboratories (Indianapolis, IN). Rats were shipped to EPA on GD 1. The presence of a positive sperm plug was considered GD 0. Animals were housed individually in clear, polycarbonate cages (20 × 25 × 47 cm) lined with laboratory-grade heat-treated pine shavings (Northeastern Products, Warrensburg, NY), with a 14:10 light/dark photoperiod (lights off at 19:00) at 20–24°C. Animals were fed NIH 07 breeding diet for rats and water from a municipal supply (Durham, NC), filtered at 5 µm *ad libitum*. These studies were conducted under protocols approved by the National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee at a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Chemicals. DPeP was obtained from Sigma (St Louis, MO; CAS # 131-18-0, lot# 1431420). Purity was verified at 100% by gas chromatography with flame ionization detection. The vehicle used to deliver DPeP was laboratory-grade corn oil (Sigma; CAS 8001-30-7; lot # 126K0117; cat # C-8267) in 2.5 ml/kg body weight.

Fetal T ontogeny in control animals. Three timed-pregnant Charles River SD dams were anesthetized with CO_2 and euthanized by decapitation on each of GD 14.5–18. Embryos (GD 14.5 and 15.5)/fetuses (GD16.5–18) were removed, anesthetized via hypothermia on ice, and dissected under a dissecting microscope. All testes from a single litter (6–20 testes per litter from 3 to 10 males per litter) were removed and immediately transferred individually into 100 µl (on GD 14.5 and 15.5) or 500 µl (on GD 16.5–18) M-199 media and incubated for 3 h at 37°C as detailed in Wilson *et al.* (2004). Medium was stored at −80°C until used for T measurement. The designation of a gestational half day (14.5–17.5) refers to a necropsy performed in the afternoon (between 13:00 and 15:00 Eastern Standard Time [EST]) versus a morning necropsy (GD 18; performed between 8:00 and 10:00 EST). All necropsies were conducted within a 2-h time frame or less to avoid any potential confounding effects of fetal growth or time of day on the fetal endpoints.

Experiments with DPeP administered only on GD 17. To conform to the strain standard used by the National Institute of Environmental Health Sciences National Toxicology Program, Harlan SD rats were used in this set of experiments. The single-dose experiments herein examined fetal T production and gene expression beginning 5–6 h after maternal treatment. This time period was selected based on previous evidence that DPeP induces morphological changes in pubertal rat testes by 6 h following dosing (Foster *et al.*, 1982). In the first experiment, dams were dosed with the vehicle or 500 mg/kg DPeP (six dams per each dose group) on the morning of GD 17 to determine if a single treatment effectively reduced fetal T production at this stage of sexual differentiation. We selected this dose for the first experiment because a single dose of DBP at 500 mg/kg reduces testis T levels by more than 50% on GD 19 (Thompson *et al.*, 2005). Next, an experiment was performed to characterize the dose-related effects of DPeP on fetal testis endocrine function at GD 17.5 following a single dose. Pregnant rats were dosed orally with DPeP at 0, 300, 600, 900, or 1200 mg/kg (three dams for each dose group) on the morning of GD 17, and necropsies and testes excision/incubations were performed 5 h post-dosing. In each litter, one testis from three males was incubated to determine T production levels. All individual dose-response experiments were designed to identify the slope and the effective dose that reduced T production of gene expression by 50% (ED50) of the control values.

Experiments with DPeP administered on GD 14–18. Pregnant Harlan SD rats were gavaged daily on GD 14–18 with 0, 11, 33, 100, or 300 mg DPeP/kg/day in corn oil (three dams per dose group) to compare the dose-related effects on fetal testis function after dosing the dam for 5 days with our data from DEHP 5-day dosing and with the single-dose study (above). On GD18, all fetal necropsies were performed within a 2-h window between 08:30 and 10:30 EST, during which all testes were extracted. One testis from three males per litter were used for *ex vivo* T production incubations as above, and the remaining testes within the litter were transferred immediately into ice-cold triazol (TRI) Reagent (Sigma) and homogenized on ice using a Kontes pestle. The volume of testes homogenate was brought to 500 µl with TRI Reagent and stored at −80°C until used for RNA extraction.

Ex Vivo testicular T production. Testicular T production was measured in media using a Coat-a-Count radioimmunoassay kit for total T according to the protocol provided by the manufacturer (Siemens Healthcare Diagnostics, Deerfield, IL). The intra-assay coefficient of variation was 3.1% (based on variability of the standard curve). The interassay coefficient of variation was 13.7%. Cross-reactivity with dihydrotestosterone was 3.2%. The limit of detection (LOD) was 0.2 ng/ml for T.

Gene expression analysis. Gene expression was analyzed using reverse transcriptase real-time PCR. RNA was extracted from testis homogenates according to the TRI Reagent protocol (Sigma) and treated with DNase I (Promega, Madison, WI) to eliminate any potential genomic DNA contamination. RNA concentration was determined using Quant-iT RiboGreen RNA Reagent and Kit (Molecular Probes, Carlsbad, CA) according to manufacturer's instructions. Complementary DNA (cDNA) was synthesized from each RNA sample using ImProm-II Reverse Transcriptase and a random hexamer primer (Promega).

For PCR analysis, gene products for insl3, StAR, and Cyp11a were amplified and detected using 5′-nuclease PCR with primer/probe sets synthesized by Integrated DNA Technologies (Coralville, IA). Primer and probe sequences were previously reported (Howdeshell *et al.*, 2007b). A total volume of 50 µl for each gene was comprised of 50 ng of cDNA, 1× PCR buffer, 0.4mM each deoxyribonucleotide triphosphate, 4 (insl3) or 8µM (StAR and CypAll) of MgCl$_2$, 12 pmol each of forward and reverse primer, 1.25 pmol fluorescent probe, 0.5 U Platinum Taq DNA Polymerase (Invitrogen) bound to Taq antibody (Life Tech), and nuclease-free water (Promega).

An internal standard curve was included in each real-time PCR assay and was used to determine absolute starting quantity (SQ) of the cDNA sample. PCR cycling parameters were as follows: initial denature at 95°C for 3 min, followed by denature at 95°C for 15 s, anneal at 56°C for 20 s, extend at 72°C for 10 s, repeated for 40 cycles, and final extension at 72°C for 10 min. Samples and standard were run in duplicate on a single plate for each assay.

Effects of DPeP on early postnatal development. Pregnant Harlan SD rats were gavaged daily on GD 8–18 with 0, 11, 33, 100, or 300 mg/kg/day DPeP in corn oil (five dams per dose group). This dosing interval covers the entire gestational period during which male offspring are sensitive to phthalate disruption of T production (Carruthers and Foster, 2005). Offspring were weighed, and AGD was measured using a dissecting microscope on postnatal day (PND) 2 (day of birth = PND 1) as previously described (Gray *et al.*, 1999). AGD was defined as the distance between the rostral end of the anal opening and the base of the genital papilla. On PND 13, male offspring were assessed for areola or nipple retention. Male offspring were weaned on PND 24 and housed in groups of two to three per cage to be maintained until further postnatal analysis. Following weaning, dams were euthanized and the number of uterine implantation sites was recorded. Postimplantation loss was determined as the number of uterine implants for a single litter minus the number of live pups at PND 2.

Statistics. Data analysis was performed using one-way ANOVA through the general linear model procedure in the Statistical Analysis System (SAS, SAS Institute, Cary, NC). If the overall ANOVA was significant at $p < 0.05$, the significant differences between control and treated groups were determined by a *post hoc* two-tailed *t*-test (LSMEANS on SAS) between litter means. Maternal data were analyzed using individual values, whereas fetal T production data were analyzed using litter mean values generated from the individual testis incubations from males within a litter. Data also were analyzed using litter means for AGD and male nipple retention. Gene expression data (messenger RNA [mRNA] SQ) were analyzed as measured based upon a single pooled sample from each litter following log10 transformation to correct for heterogeneity of variance.

Dose-response curves from all experiments were analyzed using untransformed and transformed (to percentage of control) data in a nonlinear four-parameter regression analysis (sigmoidal fit with variable slope Prism GraphPad 5.01 software, GraphPad Software, Inc., La Jolla, CA). For logistic regression analyses, the control dose value was set to 1 mg/kg/day rather than 0 mg/kg/day so the control data would be included in the analysis as Prism converts the dose to log10 values (the log of dose zero does not exist, whereas the log value of the control of one is zero). Differences between dose-response curves were determined using sum of squares *F*-test for the ED50 parameter and was considered significant at $p < 0.05$ using Prism software. Relative potency factor calculations between DEHP data derived from previous studies (Gray *et al.*, 2009; Howdeshell *et al.*, 2008) and DPeP endpoints were made by dividing the ED50 of the reference compound (DEHP) by the ED50 of the test compound (DPeP).

RESULTS

Maternal and Litter Effects

Overt maternal and fetal toxicity was evaluated in all experiments by measurement of maternal body weight gain and pup or fetal survival, respectively. Administration of a single dose of DPeP at doses as high as 1200 mg/kg did not induce any overt maternal or fetal toxicity 5 h after dosing. Five-day (GD 14–18) DPeP dosing did not cause any overt maternal or fetal toxicity at dosage levels as high as 300 mg/kg/day (data not shown).

Administration of DPeP for 10 days (GD 8–18) resulted in a slight reduction in total maternal weight gain between GD 8 and 18 from 40 ± 3.7 g in controls to 35 ± 0.6 g in the 300 mg/kg/day group (not significant, $F = 0.9$, $p = 0.49$). However,

increasing dosage levels of DPeP resulted in a significant increase in the F1 fetal/pup mortality rate (numbers of implants minus numbers of viable 2-day-old pups) with just $5.6 \pm 3.4\%$ in the control group compared with $51.6 \pm 15\%$ loss in the highest dose group (300 mg/kg/day) ($p < 0.01$). Regression analysis of pup survival from implantation to PND 2 (as a percentage of control) yielded an ED50 of 294.3 mg/kg/day DPeP (Fig. 7). The mean number of pups on PND 2 in the control group was 13.0 ± 0.5, whereas the mean number of pups in the 300 mg/kg/day DPeP dose group was 6.6 ± 2.4. There was no significant reduction in offspring weight on PND 2 (control $= 7.8 \pm 0.1$ g vs. 300 mg/kg/day $= 6.9 \pm 0.3$ g [$p = 0.12$, t-test]).

Fetal T Ontogeny and DPeP Single Dose

Because GD 16.5 was the earliest fetal age at which we could detect a significant level of T production by the incubated testis (Fig. 1), subsequent experiments examined the effects of a single acute dose of DPeP on T production on GD 17. This GD was selected to determine if DPeP significantly reduces testicular T production 24 h after the earliest detection of significant T production. In the next experiment, we determined that a single maternal dose of 500 mg/kg/day significantly decreased fetal testicular T production 5 h after dosing (< 0.0001, Fig. 2).

Ex Vivo Fetal Testicular T Production Dose-Response

Administration of a single dose of DPeP to pregnant dams on GD 17 resulted in dose-related decreases in fetal T production, 5 h following dosing. Similarly, DPeP administered to pregnant dams over the period of sexual differentiation (GD 14–18) decreased fetal testicular T production on GD 18 in a dose-responsive fashion. The 1-day exposure significantly reduced T production at dose levels of 300 mg/kg/day or higher (< 0.05), whereas the 5-day exposure significantly reduced T production at dose levels of 33 mg/kg/day or higher (< 0.05,

FIG. 2. Comparison of fetal testicular *ex vivo* T production after 3 h incubation between rats exposed *in utero* to vehicle or 500 mg/kg DPeP for 5 h on GD 17. Columns represent mean value ($\pm$ SEM) of all testes from six litters. Asterisk (*) represents statistical significance from control as determined by ANOVA, two-tailed t-test ($p < 0.0001$).

Fig. 3a). The ED50 for DPeP 5-day exposure on fetal T production was 47.7 mg DPeP/kg/day, whereas DEHP 10-day *in utero* exposure yielded an ED50 of 388.2 mg DEHP/kg/day (Fig. 3b).

Fetal Testis Gene Expression

Five-day DPeP exposure reduced fetal testicular mRNA expression levels of all three genes tested in the current study (Figs. 4a–c). Total mRNA copies of StAR, insl3, and Cyp11a were significantly reduced from control values at dose levels of 100 mg/kg/day or greater (< 0.05) on GD 18. DPeP reduction of

FIG. 3. *Ex vivo* fetal testicular T production following a single maternal dose (DPeP GD 17.5) or 5 days of maternal dosing (DPeP 14–18) (Panel A). (Panel B) shows a comparison between the dose-response curves for DPeP (solid line) and 10-day maternal dosing with DEHP (GD 8–18, dashed line, data derived from Howdeshell *et al.*, 2008). Data points represent litter means ($\pm$ SEM) for two to three litters (DPeP) or three to six litters (DEHP). The ED50's for each curve are 666.6 mg/kg/day DPeP GD 17, 47.4 mg/kg/day DpeP GD 14–18, and 388.2 DEHP 8–18.
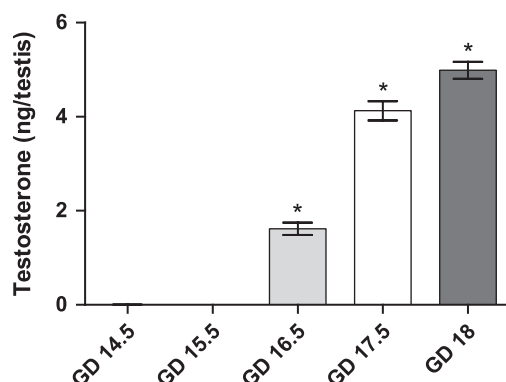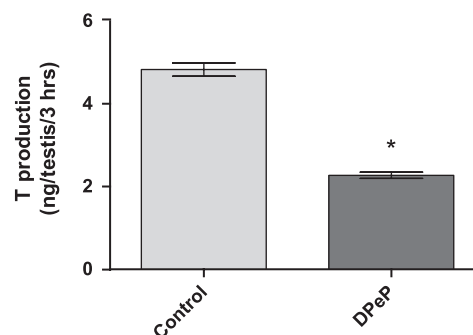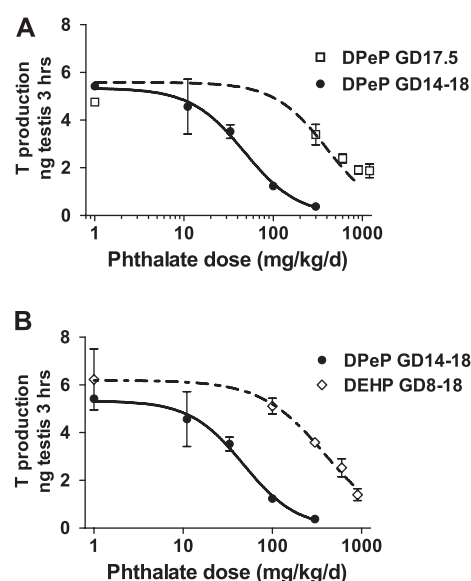
FIG. 1. Fetal testicular T production ontogeny in control rats (GD 14.5–18). Data columns represent mean value of *ex vivo* T production of all testes from two (GD 14.5) to three litters after 3 h of incubation. Error bars are $\pm$ SEM. Asterisks (*) represent statistically significant from previous GD, determined by ANOVA, two-tailed t-test ($p < 0.0001$).
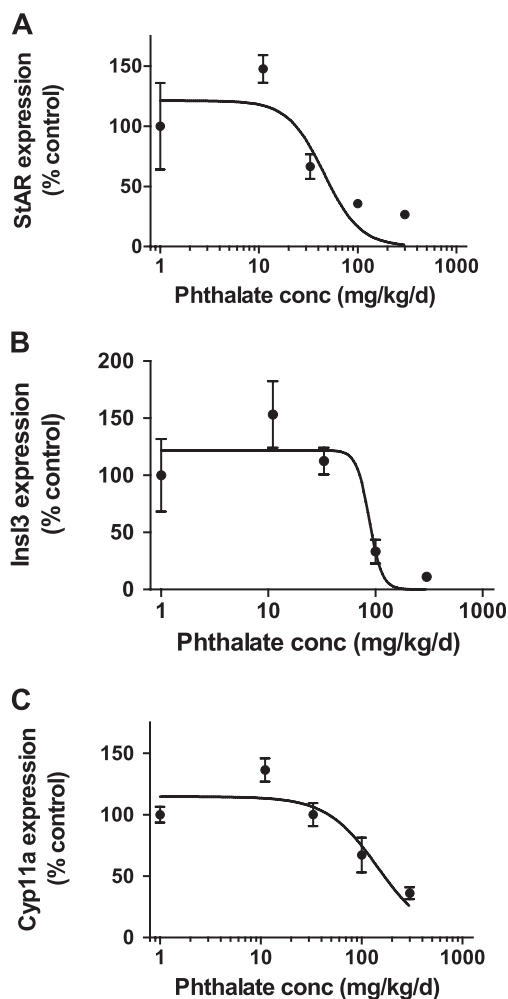
FIG. 4. Fetal testicular gene expression dose-response data for (A) StAR (ED50 = 45.65), (B) insl3 (ED50 = 87.46), and (C) Cyp11a (ED50 = 143.7), following 5-day DPeP exposure during sexual differentiation (GD14–18). Each data point represents the mean ± SEM of three to six litters.

TABLE 1

NOAEL and ED50 Values for T Production, StAR, Cyp11a, and insl3 Gene Expression following DPeP or DEHP *In Utero* Exposure during Sexual Differentiation. DEHP Prenatal Data derived from Howdeshell *et al.* (2008) and The Postnatal Data from Gray *et al.* (2009). Values Derived from Dose-response Regression Profiles in the Current Study Used three to six litters per dose group

Endpoint/treatment	ED50 mg/kg/day	NOAEL
T production		
DPeP 1 day (GD 17.5)	667	300
T production		
DPeP 5 days (GD 14–18)	47	11
DEHP 10 days (GD 8–18)	388.2	100
StAR mRNA expression		
DPeP 5 days (GD 14–18)	46	33
insl3 mRNA expression		
DPeP 5 days (GD 14–18)	87	33
Cyp11a mRNA expression		
DPeP 5 days (GD 14–18)	143	100
DPeP 5-day neonatal and infantile endpoints		
AGD at day 2	257	33
Retained nipples/areolae	175	100
Reduced viability	294	33
DEHP neonatal and infantile endpoints		
AGD at day 3	521	300
Retained nipples/areolae	785	300
Reduced viability	ND	ND

Note. ND, not determined.

all three genes had equal (StAR) or higher (insl3 and Cyp11a) ED50s and higher no observable adverse effect levels (NOAELs) than did DPeP reductions of fetal T production (Table 1).

Early Postnatal Effects of DPeP

Following 10 days (GD 8–18) of *in utero* exposure, DPeP significantly reduced PND 2 male offspring AGD at a dose of 100 mg/kg/day and greater ($p < 0.05$, Fig. 5a) and induced significant male nipple retention by PND 13 at a dose of 300 mg/kg/day DPeP ($p < 0.05$, Fig. 5b). Comparison of T production data from the 5-day *in utero* exposure to AGD reduction data from the 10-day *in utero* exposure demonstrates that a reduction in T production occurs at a lower dose than does a reduction in neonatal male rat AGD. Reduction of T production by approximately 80% (at the 100 mg/kg/day dose) correlated with a 20% decline in AGD at the same dosage level (Fig. 6). Pup viability from implantation through

PND 2 was significantly decreased at dose levels of 100 mg/kg/day DPeP and greater ($p < 0.05$, Fig. 7).

DISCUSSION

This study demonstrates that DPeP reduces fetal testicular T production, StAR, Cyp11a, and insl3 gene expression levels and induces early postnatal reproductive alterations in male offspring. The potency of DPeP for inducing these changes is greater than that of previously examined antiandrogenic PEs. Potency results in the fetal male from the current study are consistent with results from an early study (Foster *et al.*, 1980), indicating that DPeP was the most potent phthalate examined in the pubertal rat model for inducing testicular injury after a 5-day dosing period. We also demonstrated testicular effects as early as 5 h following dosing, consistent with effects reported by Foster *et al.* (1982). For these reasons, we are using DPeP as a tool to study the earliest effects of PEs on fetal endocrinology and gene expression in more detail. DPeP is one of several phthalate compounds included in the U.S.EPA OCSPP's action plan and is currently on the agenda for inclusion in a cumulative risk assessment on the phthalates (U.S.EPA, 2009). Therefore, we generated dose-response data to fill a data gap for DPeP,
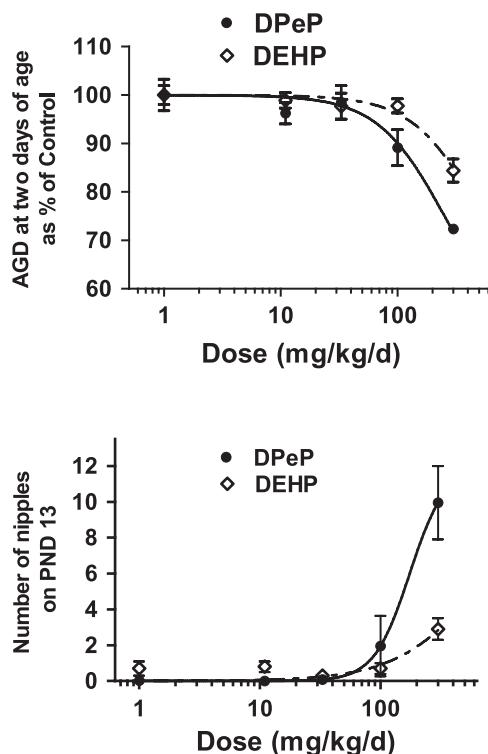
FIG. 5. Early postnatal effects of *in utero* DPeP exposure (GD8–18) and DEHP (Gray *et al.*, 2009) on (A) AGD (PND 2, ED50 = 252.3 for DPeP and 521.0 for DEHP) and (B) nipple retention (number of female-like nipples per male out of 12 possible; PND 13, ED50 = 175.0 for DPeP and 784.8 for DEHP) in SD male rats. Female AGD was approximately 50% of control males. Data points represent mean (± SEM) of four to five litters.

which would be necessary for including this compound in an antiandrogen compound cumulative risk assessment. Additionally, data generated by this study further support the hypothesis that fetal testicular T production is a more sensitive endpoint for the antiandrogenic action of phthalate compounds than are genomic and early postnatal endpoints. T production may therefore be the most appropriate critical effect to consider in the risk assessment process.

In both humans and rodents, testicular androgen production begins during the sexual differentiation period and is partially
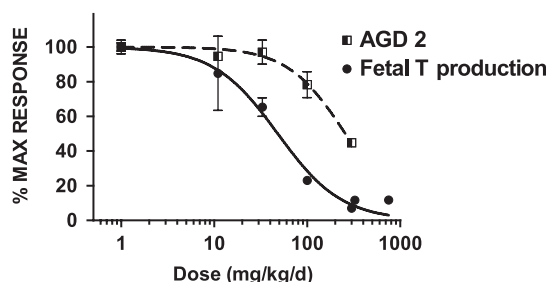

FIG. 6. Male AGD decline on PND 2 following *in utero* exposure to DPeP from GD 8 to 18 (ED50 = 252.3) versus fetal testicular T production on GD18 following *in utero* exposure to DPeP from GD 14 to 18 (ED50 = 47.0). Each data value represents the litter means of three to six litters.
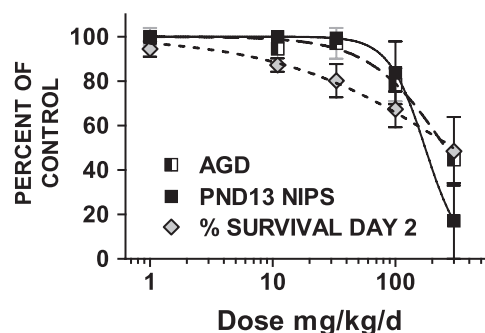

FIG. 7. Dose-responsive decline of percentage of males without nipples at PND 13 (100% Normal = 0 nipples vs. 0% normal = 12 nipples; ED50 = 175.0) versus decline in male AGD at PND 2 (Control male AGD = 100% vs. control female AGD = 0% [50% of control male value] ED50 = 252.3) and decline in pup survival from implantation to PND 2 (ED50 = 294.3). Data points represent litter means (± SEM) of four to five litters.

responsible for masculinization of male reproductive features including differentiation of the Wolffian ducts into the epididymis, lengthening of the AGD, and formation of the vas deferens, seminal vesicles, and external genitalia. Several rodent studies have demonstrated that phthalate exposure throughout this critical period for male development inhibits testicular androgen production (Howdeshell *et al.*, 2008; Lehmann *et al.*, 2004; Parks *et al.*, 2000; Shultz *et al.*, 2001) and thereby interferes with organizational development of these features (Barlow and Foster, 2003; Foster, 2006). Blocking androgen-driven masculinization during this critical programming period (GD 14–18) has more impact on reproductive tract differentiation than does blocking androgen action on later morphological differentiation (after GD 18) (Welsh *et al.*, 2008) with antiandrogen treatments on GD 16–18 being more effective than earlier or later in gestation (Wolf *et al.*, 2000; Carruthers and Foster 2005). However, the mechanism behind phthalate-induced disruption of T production within this window is unclear. Existing evidence of the mechanism driving phthalate-induced hormone decline relates to exposure-induced abnormal Leydig cell aggregation (Mahood *et al.*, 2005; Mylchreest *et al.*, 2002) and declines in gene expression of *insl3* (Wilson *et al.*, 2004) and several steroid synthesis-related genes (Barlow *et al.*, 2003; Howdeshell *et al.*, 2008; Johnson *et al.*, 2007; Lehmann *et al.*, 2004; Liu *et al.*, 2005; Shultz *et al.*, 2001). Because most studies have examined the effects of PEs on androgen levels late in gestation (GD 18–21), information about the earliest pathways affected by phthalates is generally lacking. In the current study, we determined that the decline in T production can be acutely induced with 5-h exposure to a high dose of DPeP on GD 17. Ongoing efforts to pinpoint the earliest time point for phthalate disruption of T production within the critical programming window are important for further investigations related to the mechanism of action for the organizational toxicity of phthalates. A comparison of DPeP gene expression dose-response data for Cyp11a, StAR, and insl3 generated in this study to similar data for DEHP recently generated in our

laboratory indicates that DPeP reduces expression of these genes with approximately three- to sixfold greater potency (Hannas, Howdeshell, Lambright, Furr, Gray, and Wilson, unpublished data). Therefore, DPeP is likely a good model phthalate for performing mechanistic studies at the molecular level that require robust responses. A comparison of the ED50s derived from the regression analyses for DPeP 5-day dosing and DEHP 10-day (GD 8–18) dosing data (Howdeshell et al., 2008) demonstrates a nearly eightfold greater potency for the ability of DPeP to decrease fetal testicular T production on GD 18 as compared with DEHP (Fig. 3b). Comparison of the ED50s for postnatal effects indicates that DPeP is approximately twofold more potent than DEHP for reducing AGD and 4.5-fold more potent for inducing male nipple retention (Gray et al., 2009). It should be noted that the current study was conducted with Harlan SD rats, whereas the studies by Gray et al. (2009) and Howdeshell et al. (2008) used SD rats from Charles River Laboratory. We conducted a study in the Harlan SD rat in which DEHP treatment of pregnant dams resulted in comparable fetal testicular T production and postnatal AGD and nipple retention outcomes to the Charles River SD (unpublished data). Therefore, it is unlikely that the increased potency of DPeP detected in the Harlan SD is solely driven by different sensitivity of the Harlan and Charles River rats to phthalate exposure.

The consistency in DPeP potency from fetal endpoints to postnatal effects supports the hypothesis that fetal declines in androgen production are causally linked to postnatal malformations in androgen-dependent tissues. We were able to accurately predict the relative magnitude of early postnatal effects (reduced AGD and nipple retention) based on the potency of DPeP for reducing fetal testicular T production relative to DEHP. Consequently, this study lends support to the notion of using fetal testicular T production decline as the critical effect for setting reference dose values in the risk assessment process.

The data collected in this study suggest that the reduction in T production would be a more sensitive critical endpoint in phthalate risk assessment than changes in gene expression data. In the U.S.EPA draft assessment of DBP, use of fetal T production as the critical effect endpoint (U.S.EPA, 2006) fostered criticism claiming that gene endpoints should alternatively have been considered as the critical effects in the DBP phthalate risk assessment because they are disrupted at lower doses of DBP than T production and would therefore result in lower NOAEL or benchmark dose levels (Janssen, 2006). In the current study, however, the resulting order of endpoint sensitivity to in utero DPeP exposure was T production = StAR expression > insl3 expression > Cyp11a expression > retained nipples > AGD. A reduction in T production integrates the smaller individual reductions of the steroidogenic enzyme gene expression. In addition, T production typically is less variable than changes in fetal testis gene expression, and as a consequence, the NOAEL value for T production is usually below those for StAR, insl3, and Cyp11a mRNA. These results

agree with the order of endpoint sensitivity identified in dose-response studies involving DBP (Howdeshell et al., 2010 in prep), BBP (Howdeshell et al., 2008), and DEHP (Hannas, Howdeshell, Lambright, Furr, Gray, and Wilson, unpublished data). Currently, we are measuring gene expression levels for these genes, and several others, using a custom PCR array platform with 96 genes per plate to determine if this method provides more accurate and precise results than the methods used herein to determine gene expression levels.

AGD has become a widely accepted endpoint to identify the antiandrogenic activity of a compound administered during the sexual differentiation period (Foster et al., 2000, Gray et al., 2000, Nagao et al., 2000, Parks et al., 2000, Tyl et al., 2004). Normally, androgens secreted by fetal testis during development lengthen the AGD in males compared with females. However, phthalate disruption of testicular androgen production during sexual differentiation in males results in decreased AGD on PND 1–2. As seen with the antiandrogenic compounds linuron, flutamide, and finasteride and the phthalates DEHP and DBP, this early decline in AGD length relative to untreated rats can be permanent through adulthood (Barlow and Foster 2003; Bowman et al., 2003; Hotchkiss et al., 2004; McIntyre et al., 2001, 2002) and males with shorter AGDs and retained female-like nipples have a higher probability of displaying severe reproductive tract malformations than do males with normal AGD lengths. Similarly, disruption of androgen action by phthalates during the male masculinization process results in retention of nipples through adulthood (Barlow and Foster 2003; Gray et al., 1999; Hotchkiss et al., 2004). In the current study, a reduction in AGD on PND 2 and nipple retention on PND 13 were seen when fetal T production was reduced by approximately 80% on GD 18. It should be noted that use of T production data as a point of departure for predicting postnatal malformations would require further investigation with additional phthalate compounds, doses, and litters. However, results of the current study indicate that the fetal and postnatal effects of DPeP exposure evaluated in this study are causally linked and levels of reduced fetal T production are predictive of adverse postnatal outcomes. All male offspring from the DPeP GD 8–18 exposures performed in this study will be assessed further in the future for later postnatal reproductive malformations including delay in preputial separation, reduction in androgen-dependent organ weights, and histopathology of the testes and epididymides. These assessments will provide further information for making potency comparisons to other phthalates and correlations with T production levels.

In the current study, the ED50 for decline in pup survival to PND 2 following in utero DPeP exposure was similar to that for inducing AGD decline. Offspring mortality has not been detected previously at a comparable dose (which would induce postnatal malformations) with other PEs (Foster, 2006; Gray et al., 2000, 2009; Howdeshell et al., 2007b). Howdeshell et al. (2008) demonstrated that maternal oral doses of 300, 600, and

900 mg/kg/day DPeP administered for 10 days (GD 8–18) resulted in complete loss of litters. Clearly, DPeP is also acting simultaneously through other mechanisms beyond antiandrogen toxicity to induce overt offspring toxicity.

Although very little toxicity or exposure data are available for DPeP, this phthalate consistently ranks highest in potency of tested phthalates for inducing male reproductive toxicity (Benson, 2009; Foster et al., 1980; Howdeshell et al., 2008). Although current levels of usage, production, and import of DPeP in the United States are unknown (U.S.EPA, 2009), Silva et al. (2010) of the Center for Disease Control recently reported that mono(4-hydroxypentyl) phthalate (a urinary biomarker of DPeP exposure) was detected above the LOD in 29% of the samples of human urine at concentrations ranging from < LOD to 8 ng/ml. The EPA action plan for phthalate risk assessment states that the agency may consider a requirement for manufacturers and processors of DPeP to notify the agency before taking action. Based on the results provided by the current study and previous studies characterizing DPeP as the most potent phthalate for inducing male reproductive effects, it would be prudent for the toxicity of this particular phthalate to be considered before increases in production and usage are approved to assure that usage would not result in exposure to potentially hazardous levels of DPeP. The toxicity threat posed by the increased potency of this phthalate in comparison to other antiandrogenic phthalates was further demonstrated in a cumulative phthalate toxicity study. Howdeshell et al. (2008) determined that DPeP contributes to dose-additive phthalate mixture toxicity and also carried the largest burden within the mixture when compared with other phthalates within the mixture. Accordingly, the hazard associated with exposure to this compound is greater than for other phthalates that were examined.

Finally, this study was designed to characterize the dose-response relationships between DPeP exposure and fetal and neonatal androgen-dependent endpoints and to identify the ED50 for each endpoint to determine the relative potency of DPeP. In doing so, we determined that a smaller sample size (3–6 litters in most cases) was appropriate for obtaining a robust sigmoidal fit for the T production dose-response, with small variability between litters at each dose for most endpoints. A larger sample size or more precise methodology could improve the strength of the gene expression data, as these endpoints tend to vary more between litters than does T production. It should be noted that whereas the design of the current study produced data that would likely be useful for risk assessment, an alternative design that incorporates more litters per dose group would be better suited for determination of NOAELs. Alternatively, a benchmark dose analysis (BMD) would likely be more appropriate for the data we report in this study for risk assessment than NOAEL determinations because BMD analyses rely more heavily on the shape of the dose-response curve than statistical determination of the lowest dose that differs from the control group.

The results of the current study are noteworthy for three main reasons. First, we provide critical data for DPeP that are needed by the U.S.EPA and the Consumer Products Safety Commission to be able to appropriately assess the toxicity hazard associated with this compound. Second, we provide evidence supporting the use of T production as critical endpoint for risk assessment. And third, we have identified an antiandrogenic phthalate that can serve as a strong model for mechanistic investigations. The high potency and strong link between fetal and postnatal antiandrogenic endpoints associated with DPeP will facilitate future investigations related to the proximate mechanism of phthalate toxicity. Rodent studies focusing on these investigations would be applicable to humans because of a highly conserved androgen pathway, which is crucial to healthy reproductive development during sexual differentiation in both species. Overall, the results of the current study with DPeP have the potential to significantly advance our understanding of phthalate risk to human male reproductive health.

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